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FOREWORD

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SUMMARY

Biomonitoring of exposure to the insecticide permethrin is usually performed by analysis of its urinary metabolite 3-phenoxybenzoic acid (3-PBA). However, chronic low-level exposures and cumulative exposures cannot be assessed by analyzing urinary biomarkers. We are engaged in the development of a methodology to assess the cumulative internal dose of exposure to permethrin, which is based on the assumption that (reactive) glucuronide conjugates of the major permethrin metabolites 3-PBA and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis/trans-Cl₂CA) will form persistent adducts to proteins, in analogy with the glucuronide conjugates of structurally related drugs. In the first year of the project the 3-PBA and Cl₂CA glucuronide metabolites of permethrin have been successfully chemically synthesized. Their identities have been assessed by means of ¹H-NMR spectroscopy and LC tandem mass spectrometry. The reactivity of these metabolites with various amino acids, peptides and albumin has been studied; various distinct adducts could be identified by LC tandem mass spectrometry. It is envisaged that the obtained results will form a firm basis for development of an adduct-based methodology for biomonitoring exposure to permethrin.

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LIST OF ABBREVIATIONS

Cl ₂ CA	3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
Diad	Diisopropyl azodicarboxylate
DMF	<i>N,N</i> -Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
HATU	<i>O</i> -(7-Azabenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
MeOH	Methanol
NMM	N-methylmorpholine
NSAID	Non-Steroidal Anti-Inflammatory Drugs
3-PBA	3-Phenoxybenzoic acid
Pd(PPh ₃) ₄	Tetrakis(triphenylphosphine)palladium(0)
PPh ₃	Triphenylphosphine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Z-Lys	N- α -Benzyloxycarbonyl-L-lysine

I INTRODUCTION

The pyrethroid permethrin is one of the most widely used insecticides. It is effective in the control of ticks, mites and lice, while having little adverse effects in humans. Its toxicity in insects, and in humans, is based on binding to sodium channels in the nervous system, leading to prolongation of the depolarizing after-potential, repetitive after-discharges and hyperexcitation (Narahashi, 2002). Recently, it has been argued that voltage-sensitive calcium channels may also be targets of pyrethroid action (Shafer and Meyer, 2004). Permethrin has been used extensively by the allied troops in the Gulf War and in operation Iraqi Freedom, *e.g.*, by impregnating it into battle dress uniforms and bed nettings. In this way permethrin can be absorbed through the skin, while oral and respiratory exposure can also occur. Probably, soldiers can be exposed to rather high doses of permethrin by migration of the compound from clothing to the skin surface (see, *e.g.*, Snodgrass, 1992). Although permethrin is generally considered as a rather safe compound, a number of adverse effects have been reported. Occupationally exposed people have been reported to experience facial skin sensations. Symptoms of acute poisonings include dizziness, headache, nausea, anorexia, and fatigue. In case of heavy exposures, muscle fasciculation and altered consciousness have been reported (He et al, 1989; Aldridge, 1990). It has been shown in animal experiments that combined exposure to (high dosages) of permethrin, DEET and pyridostigmine bromide resulted in enhanced neurotoxicity, increased mortality, increased oxidative stress, and behavioral alterations (Abou-Donia et al., 1996; Abu-Qare and Abou-Donia, 2000a, 2001; Abdel-Rahman et al, 2004; for a review see Abu-Quare and Abou-Donia, 2003). On the basis thereof it was postulated that such combined exposures might have contributed to the etiology of the so-called Gulf War Illness. Therefore, careful biomonitoring of exposure to permethrin is important for the military community.

The metabolism of permethrin has been investigated in several species (Huckle et al, 1981). The major metabolites are *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*cis/trans* Cl₂CA) and 3-phenoxybenzoic acid (3-PBA; see *e.g.*, Tyler et al., 2000; Hardt & Angerer, 2003). The latter metabolite is formed in two phases (see Figure 1). First, esterase-mediated cleavage of the parent compound will give 3-phenoxybenzyl alcohol, while in the second phase this compound is oxidized enzymatically (Bast and Kampffmeyer, 1998; Heder et al, 2001) to 3-PBA. The 4'-hydroxy derivative of 3-PBA has been identified as the major metabolite of permethrin in the rat (Angerer and Ritter, 1997). Subsequently, phase II metabolism will give the respective conjugates, mostly glucuronides, which facilitate the excretion process.

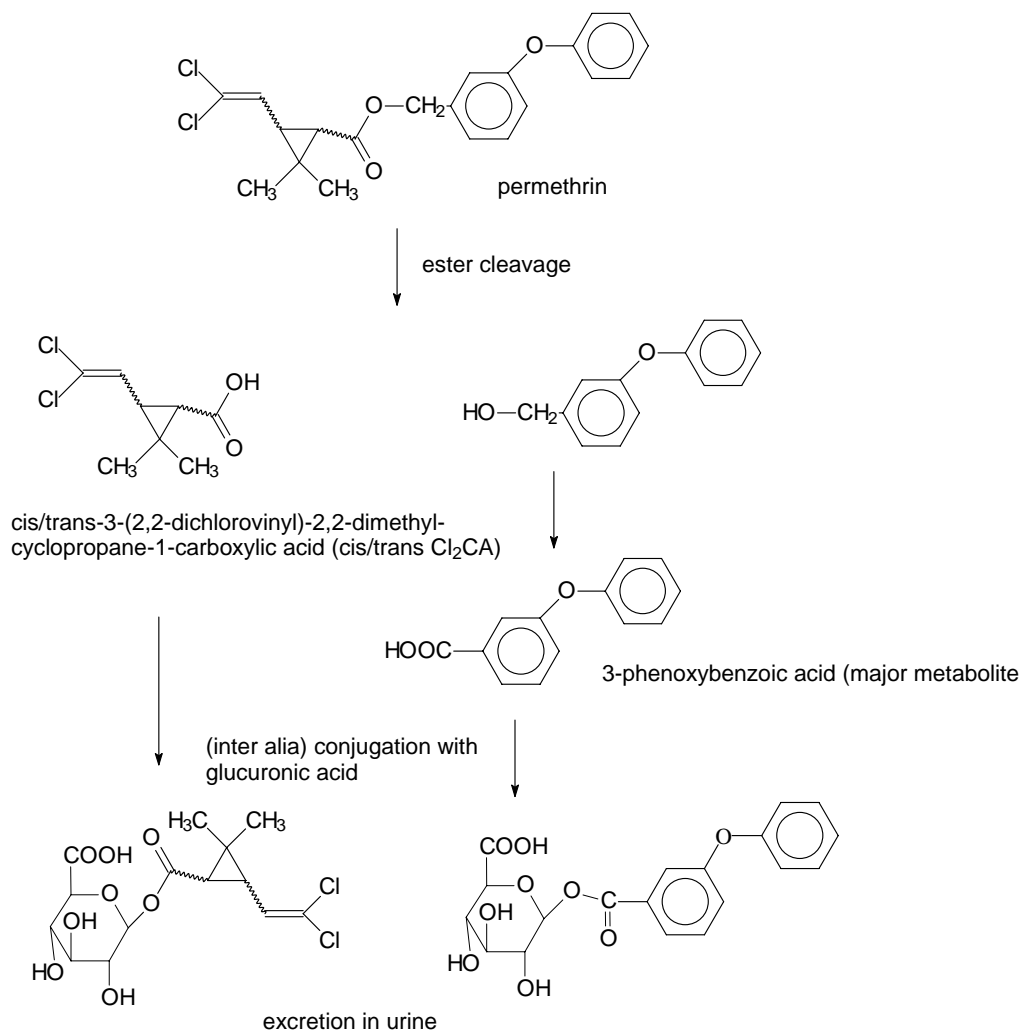


Figure 1. Metabolism of permethrin in mammals

Biomonitoring of exposure to permethrin is usually performed by analysis of its urinary metabolite 3-PBA, after acidic hydrolysis of its glucuronide (see, e.g., Hardt and Angerer, 2003; Leng et al., 2003; Abu-Qare and Abou-Donia, 2000b; Baker et al., 2004), albeit that conjugates of cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid are excreted into human urine in similar quantities as the PBA derivatives (Hardt and Angerer, 2003). Studies with a volunteer who had been exposed (orally) to the closely related pyrethroid cyfluthrin revealed that most (93%) of the urinary metabolites are excreted within 24 h (Leng et al., 1997). Consequently, urine samples for biomonitoring purposes should be taken within the first 24 h after exposure. It follows that chronic low-level exposures and cumulative doses can not be assessed by analyzing the urinary biomarkers. This observation constitutes an evident research gap.

It is well known that protein adducts of xenobiotics represent a much more persistent class of biomarkers than metabolites excreted into urine, having half lives up to several weeks or months. For instance, protein adducts in human tissues have provided mechanistic insight into the epidemiological associations between smoking and cancer (Phillips, 2002). In our

laboratory we developed methods for biomonitoring of exposure to CW agents based on mass spectrometric analysis of such protein adducts, , e.g., adducts of sulfur mustard with hemoglobin and albumin and of adducts of nerve agents with butyrylcholinesterase (for overviews see Black and Noort, 2005 and Noort et al., 2002, 2005). In view of its chemical structure, it should not be expected that permethrin itself will react with proteins to give adducts, as experimentally established for albumin (Abu-Qare and Abou-Donia, 2002).

In the current study we explore the feasibility of biomonitoring of exposure to permethrin based on the determination of long-lived protein adducts derived from metabolites of permethrin. Within this context we use the (presumed) protein adducts of glucuronides of the two major carboxylic acid metabolites of permethrin, i.e., 3-PBA and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (see Figure 1). Such adducts may serve as cumulative biomarkers for chronic exposure to permethrin, since the O-acyl glucuronides represent a unique class of electrophilic metabolites, capable of reaction with nucleophilic sites in proteins. Numerous examples of these reactions have been documented in which the O-acyl glucuronides originated from drugs having a carboxylic acid moiety, such as several non-steroid anti inflammatory drugs (NSAID's), lipid lowering agents (gemfibrozil, clofibrilic acid), diuretic agents (furosemide) and the antiepileptic drug valproic acid (Benet et al, 1993; see Bailey and Dickinson, 2003 for an extensive overview). McKinnon and Dickinson (1989) investigated the persistence of adducts of diflunisal- and probenecid-glucuronides with plasma proteins in volunteers. The adducts were still measurable at least one month after the parent drugs were undetectable. These results hold promise for the current project.

Statement of work

The work described here is focused on the development of methods for biomonitoring exposure to permethrin, which are based on long-lived adduct with proteins. This will enable biomonitoring of chronic, low-level exposures to this compound. In order to develop such methods:

1. It will be assessed whether the potentially reactive permethrin metabolites 3-phenoxybenzoic acid glucuronide (3-PBA glucuronide) and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid glucuronide (cis/trans-Cl₂CA glucuronide) can form adducts with proteins in human plasma.
2. A sensitive liquid chromatography tandem mass spectrometry procedure will be developed for the most suitable/abundant adduct with albumin, after enzymatic digestion.
3. The in vivo formation of the adduct will be further evaluated in laboratory animals. It will also be evaluated whether the adduct can be analyzed in plasma samples of US soldiers who have used permethrin extensively during deployment.

Time schedule

Year 1: 1

Year 2: 2

Year 3: 3

II MATERIALS AND INSTRUMENTATION

II.1 Materials

The following commercially available products were used:

3-Phenoxybenzoic acid (Fluka, Zwijndrecht, The Netherlands); Cl₂CA (Specs Research Laboratory, Delft, The Netherlands). Acetonitrile (Baker Chemicals, Deventer, The Netherlands); pronase Type XIV from *Streptomyces Griseus* (E.C. 3.4.24.31) (Sigma Chemical Co., St. Louis, MO, U.S.A.); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PyBOP and Fmoc-amino acids (Novabiochem); trifluoroacetic acid, trypsin (Aldrich, Brussels, Belgium).

Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10 kDa filters were obtained from Schleicher & Schuell (Dassel, Germany). Ultrafree (100 kD molecular weight cut-off; 15 ml) centrifugal ultrafilters were obtained from Millipore (Bedford, MA). Albumin affinity chromatography was carried out on HiTrap Blue HP columns (1 ml; Amersham Biosciences, Uppsala, Sweden). Desalting of albumin fractions was carried out on PD-10 columns containing Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden).

II.2 Instrumentation/devices

Peptide synthesis

Solid phase peptide synthesis was carried out on a Syro 2000 (MultisynTech, Germany) peptide synthesizer on a 10 µmol scale, using commercially available amino acids and customized Fmoc-based protocols. After synthesis, the peptides were split off from the resin and purified to homogeneity with semi-preparative HPLC.

Liquid chromatography experiments were run on an ÄKTA explorer chromatography system (Amersham Pharmacia, Uppsala, Sweden). Columns used were a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden), a Zorbax SB C-18 column (4.6 mm x 150mm; 5 µm, Zorbax, Mac-Mod Analytical, Chadds Ford, PA, USA) and a Source 15 RPC column (Amersham Pharmacia, Uppsala, Sweden).

LC/electrospray tandem mass spectrometric analyses for obtaining structural were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Accurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C18 (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H₂O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10⁻⁴ mBar).

Other mass spectrometric analyses were carried out on a TSQ Quantum Ultra mass spectrometer (Finnigan, Thermo Electron Corporations, San Jose, USA) equipped with an Acquity Sample Manager and Binary Solvent Manager (Waters, Milford, USA). For LC-MS experiments, the liquid chromatograph was connected to the mass spectrometer source via the Sample Manager equipped with a 10 µl loop and an Acquity BEH C18 column (1.7 µ particles, 1 x 100 mm; Waters, Milford, USA). The liquid chromatography system was run with a 25 minute linear gradient from 100% A to A/B 55.5/44.5 v/v (A: 0.2% formic acid in water; B: 0.2% formic acid in acetonitrile) at a flow rate of 0.09 ml/min. The TSQ Quantum Ultra mass spectrometer was operated with a spray voltage of 3 kV, a source CID of 0 V, a sheath gas pressure of 41 A.U., aux gas pressure of 2 A.U. and a capillary temperature of 350 °C. Positive electrospray product ion spectra were recorded at an indicated collision energy of

15-20 eV, using argon as the collision gas at a pressure of 1.5 mTorr. Negative electrospray product ion SRM data was recorded at an indicated collision energy of 15-20 eV.

¹H-NMR spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) VXR 400S spectrometer operating at 400.0 MHz respectively. Chemical shifts are given in ppm relative to tetramethylsilane. The solvent signals at 2.525 ppm (residual Me₂SO-*d*₅ in Me₂SO-*d*₆) or 7.260 ppm (residual CHCl₃ in CDCl₃) served as a reference.

III EXPERIMENTAL PROCEDURES

Allyl α/β -D-Glucopyranuronate (1)

To a solution of D-glucuronic acid (4 g, 20.6 mmol) in 40 ml of DMF was added DBU (3.4 ml, 22.6 mmol) at 25°C. The mixture was stirred for 15 minutes, and subsequently allyl bromide (2.2 ml, 24.6 mmol) was added. The mixture was stirred over the weekend at room temperature. The solvent was then removed under high vacuum and heating. Portions of the product were purified before use in subsequent reactions by chromatography over silica gel (5-20% MeOH/DCM) and washed with DCM to remove traces of DBU.

Yield: 2.6 g of a white powder (53%). MS (ES⁻) data: 279 [M + HCOO⁻]

Allyl 1-O-(3-phenoxybenzoyl) α/β -D-glucopyranuronate (2)

To a solution of 3-phenoxybenzoic acid (473 mg, 2.04 mmol) and triphenylphosphine (540 mg, 2.04 mmol) in THF (8 ml) and DMF (1 ml) at -10°C was added DIAD (400 μ l, 2.04 mmol). After 5 min, a solution of compound (1) (170 mg, 1.04 mmol) in THF (2 ml) and DMF (0.5 ml) was added slowly over 10 minutes. After 5 h the solvent was removed under vacuum and the product purified over silica gel (0-10% EtOH/DCM). This yielded the desired product (2) as an enantiomeric mixture.

Yield 29.4 mg (9.8%) of an amorphous solid. MS (ES⁻) data: 475 [M + HCOO⁻], 213 (3-PBA). ¹H-NMR: H1, β -anomer (δ 5.7, 1H, d, J=7.74Hz); H1, α -anomer (δ 6.3; 1 H, d J=3.5Hz).

1-O-(3-phenoxybenzoyl) β -D-glucopyranuronic acid (3) (3-PBA glucuronide), first batch

To a solution of allyl ester (2) (23.6 mg, 0.05 mmol) in THF (100 μ l) at 0°C was added Pd(PPh₃)₄ (5.8 mg, 0.005mmol) followed by pyrrolidine (4.3 μ l/50 μ l THF). The mixture was stirred for 60 minutes at this temperature, and when the reaction was judged to be complete as monitored by TLC, the solvent was removed under vacuum. The product was purified by preparative HPLC and yielded the β -3-PBA glucuronide as an amorphous solid (1.1 mg, 5.6%) The β -form of the product was confirmed by ¹H-NMR (δ 5.7, 1H, d, J=7.74Hz), with no traces of α -anomer detectable. The correct mass was assessed by LC-tandem (ES-)MS: 389, [M-H]⁻, 503 [M+TFA]⁻.

Allyl 1-O-(3-phenoxybenzoyl) β -D-glucopyranuronate (2), second batch; preparation by selective acylation

3-PBA, allyl glucuronate (1) and HATU were stirred in dry acetonitrile with NMM under nitrogen at room temperature. After 1 h, the solvent was removed under vacuum and coevaporated with DCM/pentane. The product was purified over silica gel (0-10% EtOH/DCM). This yielded the desired product as a single isomer (according to HPLC; gradient elution 5-80% CH₃CN/H₂O + 0.1% TFA in 40 min). Yield: 1.37g, 80%.

1-O-(3-phenoxybenzoyl) β -D-glucopyranuronate (3), second batch

The deprotection of this batch to obtain 3-PBA acyl glucuronide (5) was performed as described above with the following quantities. Allyl ester (2) (810 mg, 1.88 mmol) Pd(PPh₃)₄ (293 mg, 0.188 mmol) and pyrrolidine (150 μ l, 1.80 mmol) in 8 ml dry THF at 0°C. After 2h, when the reaction was judged complete by HPLC, the solvent was removed under vacuum and purified over silica gel (5-50% EtOH/DCM). This yielded the product as a powder (128 mg, 17%) HPLC indicated this batch as a single isomer, also when spiked with the 3-PBA acyl glucuronide from the first synthesis batch using the other method. LC-MS(MS) confirmed the mass [M-H]⁻ = 389 and also showed a single peak in the total ion current.

¹H NMR spectroscopy showed some serious disturbances in the chemical shift region (around 5.7) where the characteristic H1-β doublet is located. This interference can be caused by solid particles in the solution or the presence of remains of allylic compounds, which is probably the source of the signals at 5.95, 5.53 and 5.48. No traces of the α-anomer were observed.

Allyl-1-O-(E/Z-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanoyl-β-D-glucopyranuronate (4)

Reaction of Cl₂CA (mixture of cis/trans; 51 mg), allyl glucuronate (55 mg) and HATU (90 mg) was carried out as described for compound **2**. The desired compound **4** was isolated by means of silica gel column chromatography. According to ¹H-NMR spectroscopy, both the α- and β-anomer were present, which could not be separated by silica gel column chromatography. Since the starting material Cl₂CA consisted of the cis and trans isomers, there are actually two α- and β-anomers, with the characteristic shifts and coupling constants. For the α-anomer: δ 6.2 and J = 3.5 Hz and for the β-anomer: δ 5.8 and J = 7.8 Hz. Between 5.9 and 6.0 the allyl multiplet was visible. With HPLC analysis two very closely-eluting peaks were visible, that could not be separated.

Mass spectrometric data (ES⁻): 469 and 471 [M + HCOO⁻]; the two chloro-isotopes were clearly visible.

1-O-(E/Z-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanoyl-α/β-D-glucopyranuronate (5)

Deprotection of compound **4** was performed as was described for compound, with the exception that the initial reaction temperature was – 80 °C. The temperature was slowly increased to 0 °C when the reaction components were thoroughly mixed. According to MS, deprotection was complete after 1 h.

Mass spectrometric data (ES⁻): 382.9 [M-H]⁻, 768.9 [2M-H]⁻.

Incubations of 3-PBA acyl glucuronide with model amino acids and peptides

Incubations (1 ml total volume) of 3-PBA glucuronide (100 μM) were performed in potassium phosphate buffer (0.05M, pH 7.4) at 37°C, for 3 h, in the presence of one of the model compounds (10 mM), Z-Lys-OH, glutathione, ASSAKQR or LKZASLQK. In addition, incubations were performed in absence of the compound or of 3-PBA glucuronide, in order to serve as control sample. Samples of the incubation mixtures were taken at different times and immediately measured by LC-MS.

Incubation of Cl₂CA glucuronide with glutathione and Z-Lys-OH

Incubation of Cl₂CA glucuronide with glutathione and Z-Lys-OH was carried out as described for 3-PBA glucuronide (see above). Only in case of glutathione, the expected adduct could be identified. According to tandem MS data, the site of modification was the thiol function.

Incubations of plasma with 3-PBA glucuronide; isolation of albumin

To 0.5 ml of human plasma was added 5 μl of a solution of 3-PBA glucuronide (**3**) in various concentrations (end concentrations in plasma: 0.5 mM, 0.05 mM, 0.005 mM and 0 mM as a control). After incubation for 2h at 37°C, 2 ml of buffer A (50 mM KH₂PO₄, pH 7.0) was added. The solutions were filtered through 45 μm acrodisc filters and albumin was isolated over a HiTrap Blue HP affinity column. This column was equilibrated with 10 ml A buffer, followed by application of the sample. The impurities were removed by flushing the column with 10 ml A buffer, followed by elution of the albumin with 3 ml B buffer (50 mM KH₂PO₄ + 1.5M KCl, pH 7.0). Between the samples the column was consecutively flushed with 5 ml

B-buffer and 20 ml A-Buffer. Subsequently, the purified albumin samples were desalted over a PD-10 desalting column, which had been equilibrated with 25 ml NH_4HCO_3 solution (50mM). After applying the samples, the columns were eluted with 3 ml NH_4HCO_3 . These albumin solutions were used for enzymatic digestion.

Pronase digestion

To 750 μl of the albumin solution was added 100 μl of pronase solution (10 mg/ml 50 mM NH_4HCO_3). This mixture was incubated for 2 h at 37°C and filtered over a 10kD filter before analysis with LC-MS(/MS).

Trypsin digestion

An aliquot (0.5 ml) of the albumin solution was lyophilized and dissolved in buffer (0.3 ml; 6 M guanidine.HCl, 100 mM Tris, 1 mM EDTA, pH 8.3). To this solution was added dithiothreitol (5 mg) and the mixture was incubated for 40 min at 55°C. Next, monoiodoacetic acid (10 mg) was added and the mixture was incubated for another 30 min at 40°C. The solutions were transferred into a Slide-a-lyzer cassette and dialyzed overnight against 50 mM NH_4HCO_3 . To the dialyzed albumin solution (\pm 3 mg albumin) was added trypsin solution (30 μl ; 1 $\mu\text{g}/\mu\text{l}$ in 50 mM NH_4HCO_3). This mixture was incubated for 4 h and filtered over a 10kD filter before analysis with LC-tandem MS.

IV RESULTS AND DISCUSSION

Synthesis of 3-PBA-glucuronide

3-PBA-glucuronide was synthesized according to two slightly different procedures (see Figure 2). The yields of the first synthesis method (Kenny et al., 2004) was very low. In this case, a mixture of α and β -anomers resulted, having the correct mass that could be separated after deprotection, i.e., removal of the allyl function. The desired β -anomer could be obtained in mg amount and could be fully characterized. A more recent method (Perry et al., 2005) was applied for synthesis of this compound. In this case, the yield was much higher and only the desired β -anomer resulted.

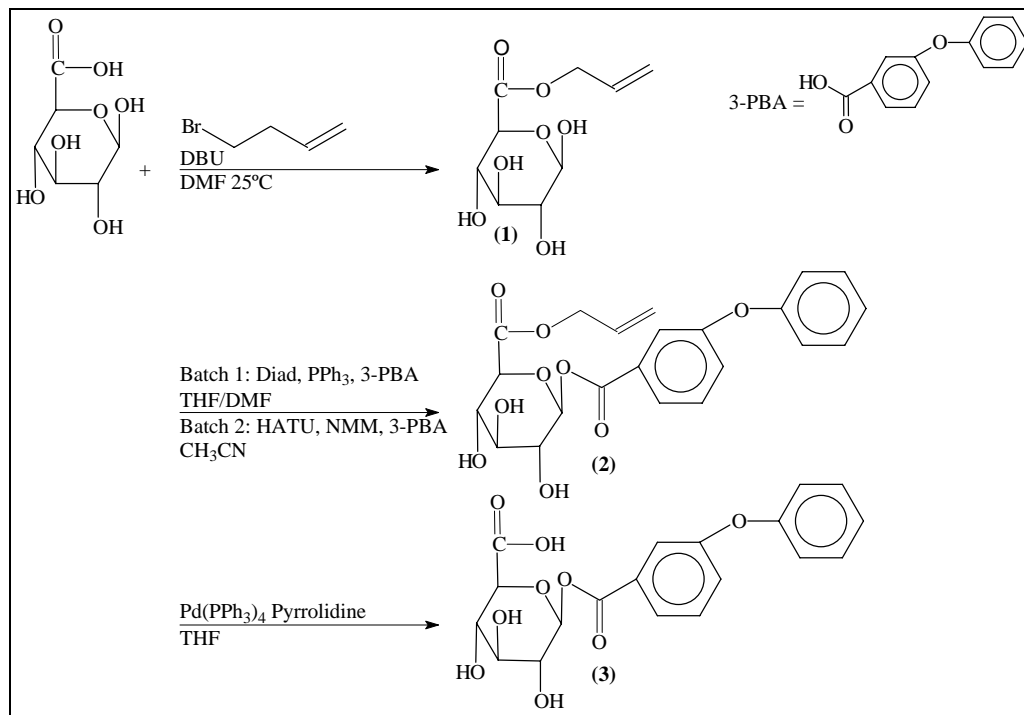


Figure 2. Synthesis of 3-PBA glucuronide (3)

Deprotection proceeded rather smoothly; the end product was purified by means of silica gel column chromatography and semi-preparative reversed phase HPLC. See Figure 3 for LC-MS and LC-tandem MS analysis; see figures 1A and 2A in appendix for NMR spectra.

Overall, the synthesis of this compound is a difficult task, which is inter alia caused by its instability. Upon storage in solution, a rapid isomerization could be observed with HPLC-UV analysis and LC-MS analysis, indicating the intrinsic lability of the compound.

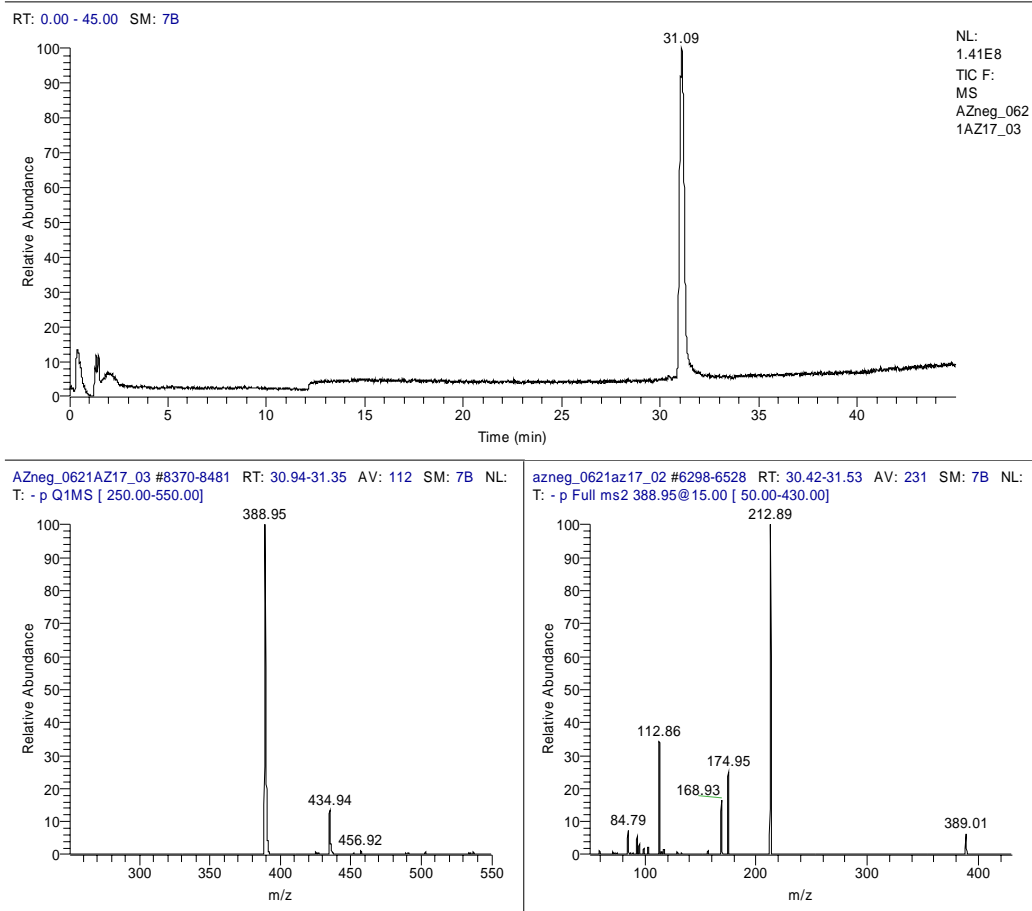


Figure 3. LC-MS and LC tandem MS spectra of 3-PBA β -glucuronide. The upper panel represents the total ion current (ES⁻). The lower, left panel is the corresponding mass spectrum; 389 [M-H]⁻; 434.9 [M + HCOO]⁻. The lower, right panel represents the tandem MS spectrum; 212.9 [3-PBA]⁻, 174.9 [389 - 3-PBA]⁻, 168.9 [3-PBA - CO₂]⁻, 112.8 [174.9 - H₂O - CO₂]⁻.

Synthesis of Cl₂CA-glucuronide

The synthesis of this acyl glucuronide (see Figure 4) was hampered by the fact that although we used a method (Perrie et al., 2005) that should result in exclusively the β -anomer (as was indeed the case for 3-PBA glucuronide), a significant amount of α -anomer resulted. Unfortunately, these isomers could not be separated, neither in the protected stage, nor in the deprotected stage. Another complicating factor in the synthesis was the fact that the end product was not longer UV-positive; therefore, following of the course of the reaction was problematic. Yet another complicating factor was the fact that Cl₂CA consists of a mixture of E and Z isomers. We decided to perform the preliminary binding experiments with the enantiomeric mixture. For mass spectrometric data: see Figure 5.

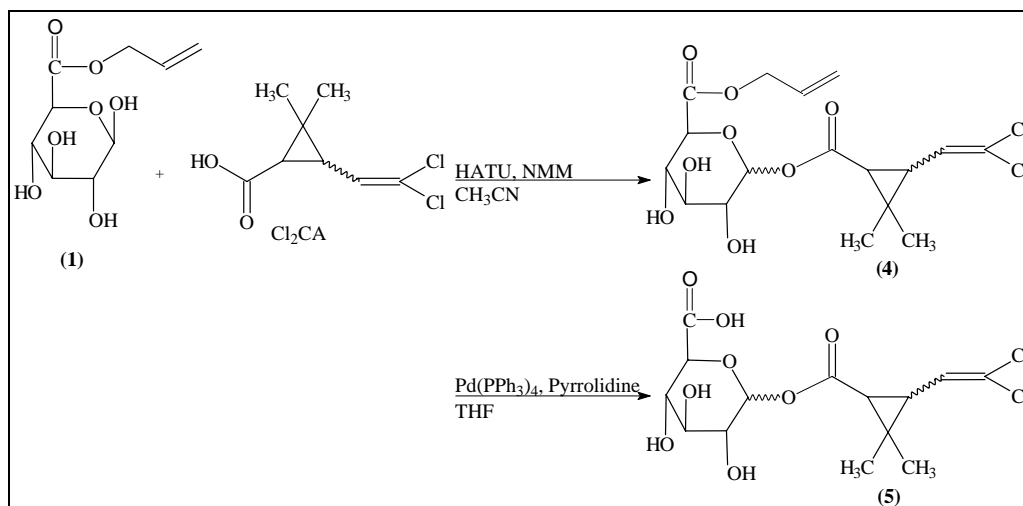


Figure 4. Synthesis of Cl₂CA-glucuronide (5)

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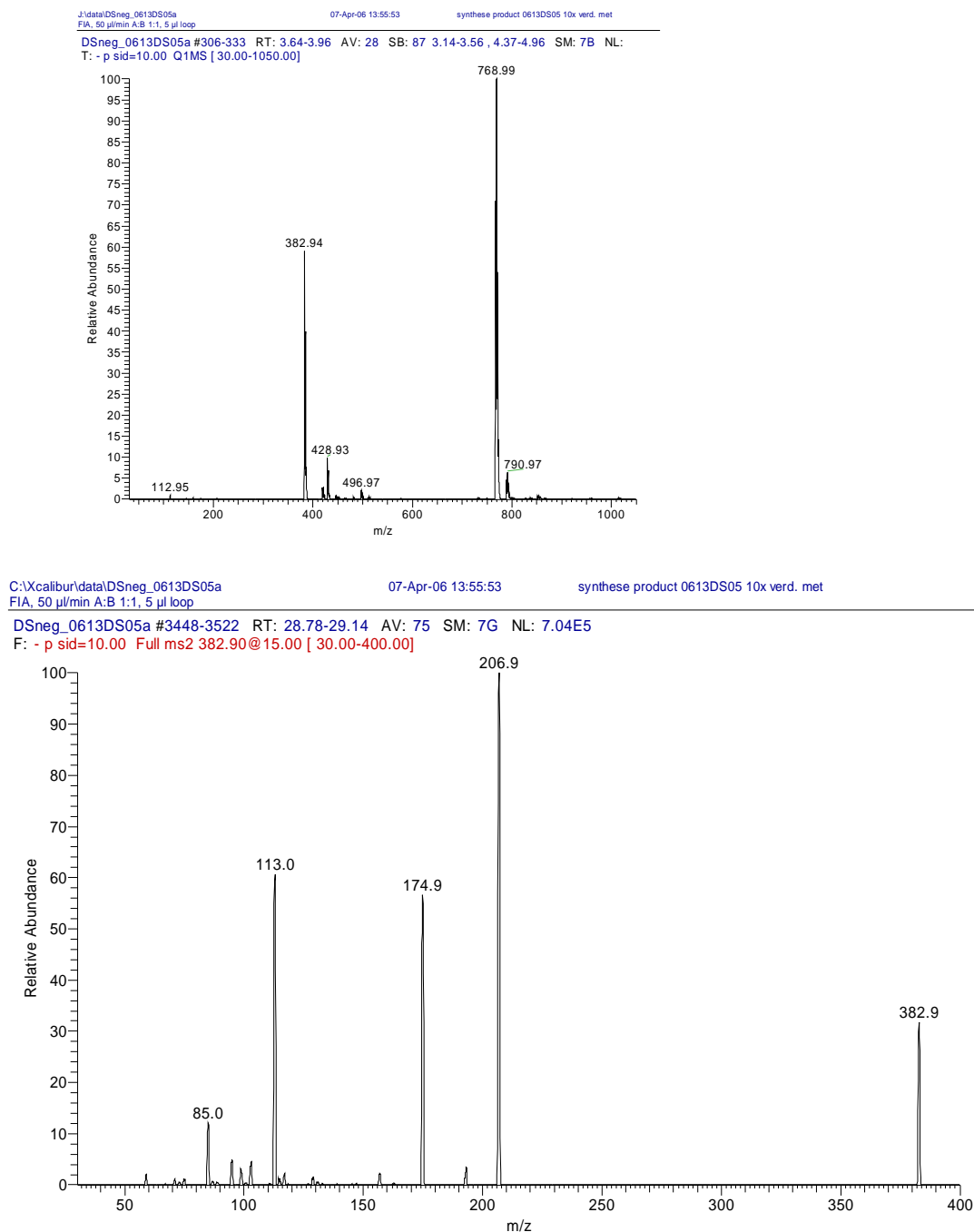


Figure 5. Mass spectrum (upper trace) of Cl_2CA glucuronide; 382.9 $[\text{M}-\text{H}]^-$, 428.9 $[\text{M}+\text{HCOO}]^-$, 768.9 $[2\text{M}-\text{H}]^-$. Tandem MS spectrum of Cl_2CA glucuronide (lower trace): 206.9 $[\text{Cl}_2\text{CA}]^-$, 174.9 $[382.9 - \text{Cl}_2\text{CA}]^-$, 113.0 $[174.9 - \text{H}_2\text{O} - \text{CO}_2]$.

Adduct formation 3-PBA-glucuronide and Cl_2CA -glucuronide with model compounds

Introduction

Conjugation to glucuronic acid (“glucuronidation”) by UDP-glucuronosyltransferase-mediated transfer of a glucuronyl moiety of UDP-glucuronic acid to a nucleophilic site of a xenobiotic is one of the major Phase II detoxification reactions. It renders the xenobiotic more polar which facilitates its excretion. This reaction takes place predominantly in the liver.

In case of glucuronidation of a carboxylic acid, potentially reactive electrophilic acyl glucuronides result that can react with nucleophilic residues within the organism.

Two mechanisms of adduct formation by O-acyl glucuronides can be distinguished. According to the *transacylation* mechanism (Figure 6), nucleophilic sites in the proteins are acylated by the O-acyl glucuronide and consequently modified with the acyl moiety derived from the “original” metabolite. E.g., in case of the glucuronide of 3-PBA this should be a 3-phenoxybenzoyl moiety (see Figure 7 for the chemical structure of an expected adduct). According to the *glycation* mechanism, an initial internal acyl migration occurs, followed by reaction with amino groups of the protein, leading to so-called Schiff base adducts (Grubb et al., 1993; Smith et al., 1990), which may eventually undergo a (slow) Amadori rearrangement (see Figure 8). However, the latter rearrangement has not been experimentally confirmed. In case of the glucuronide of 3-PBA and Cl₂CA the expected Schiff-adduct will have the chemical structure as shown in Figure 9.

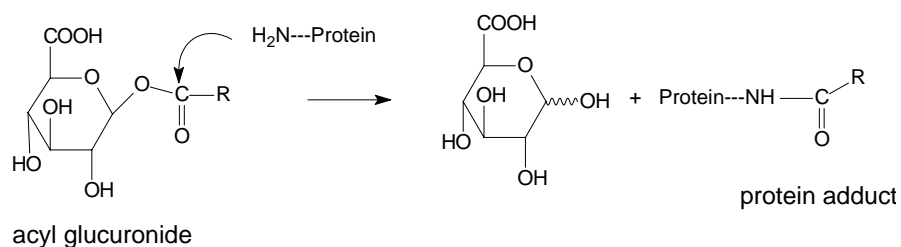


Figure 6. Adduct formation of acyl glucuronides with proteins by means of the transacylation mechanism

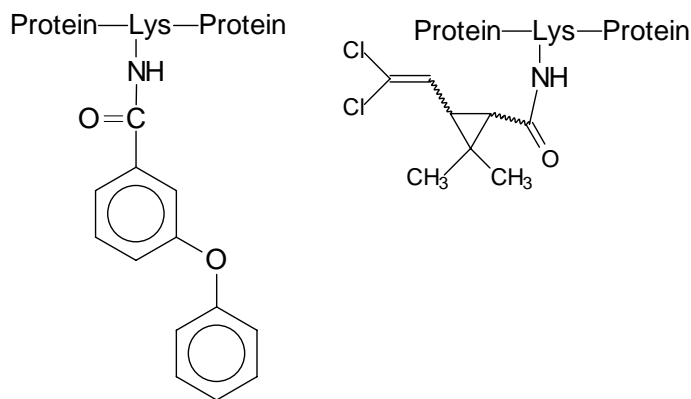


Figure 7. Schematic structure of a presumed lysine-adduct of permethrin-derived O-acyl glucuronides, according to the transacylation mechanism

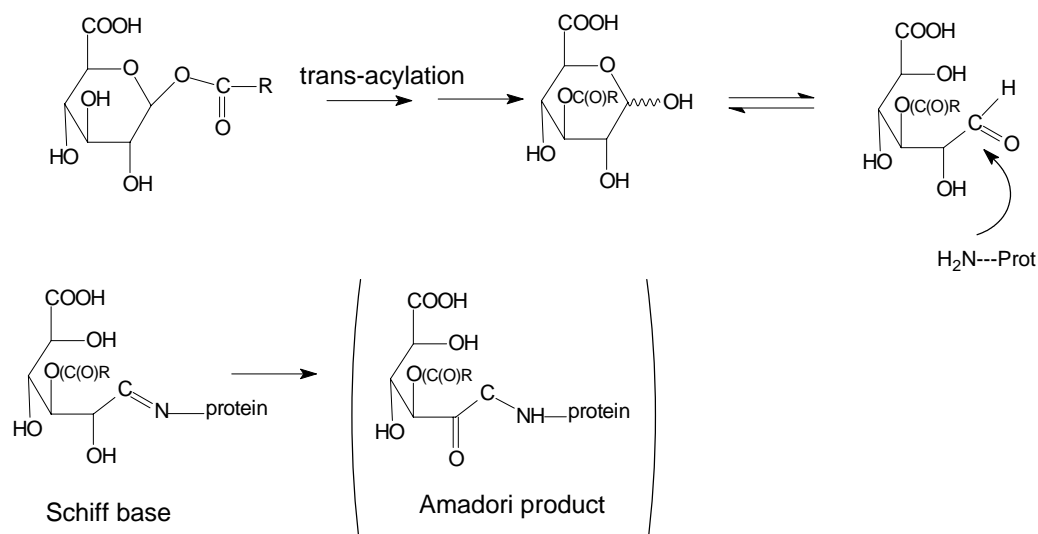


Figure 8. Adduct formation by acyl glucuronides via the glycation mechanism

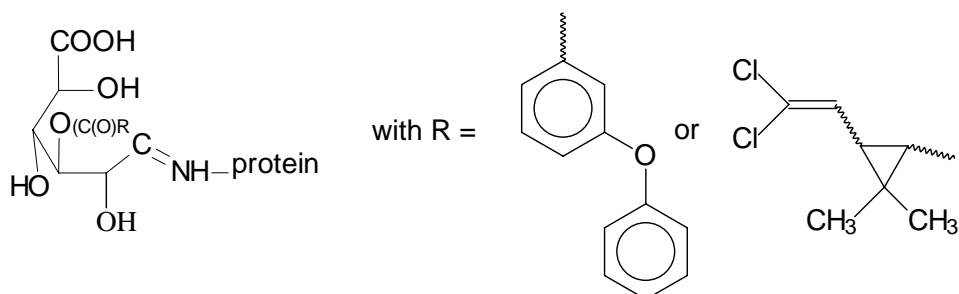


Figure 9. Identity of presumed adduct of permethrin-derived O-acyl glucuronide, according to the glycation mechanism

In general, the acyl glucuronides are stable enough to enter the blood stream (see, Sallustio et al, 2000).

The most likely candidate for protein adduct formation by acyl glucuronides is human serum albumin (HSA), which is a rather abundant protein in the plasma (see, e.g., Presle et al., 1996; Qiu et al., 1998). It has been demonstrated that the lysine 195 and 199 residues in the hydrophobic pocket of subdomain IIA of HSA are preferentially modified by various acyl glucuronides (Ding et al., 1993, 1995; Zia-Amirhosseini et al., 1995). The most extensive investigations were performed on the reactivity of the glucuronide of the NSAID tolmetin with human serum albumin, with identification of the binding sites by means of tandem mass spectrometry (Ding et al, 1995). Lysine 199 reacted not only via the Schiff base mechanism, but also by nucleophilic displacement, as did lysine 541. However, for lysine 199 the Schiff base formation predominated.

Adducts to lysine residues are probably rather stable *in vivo*. Interestingly, we have recently shown that these particular lysine residues are also highly reactive towards the acylating agent phosgene (Noort et al., 2000). In this case an intramolecular adduct was formed, in which the

lysine 195 and 199 residues were bridged intramolecularly by an urea-type chemical bond (with the carbonyl moiety derived from phosgene). It has also been demonstrated that these particular amino acid residues are modified by penicillin (Yvon et al., 1989), and that the resulting adduct is involved in allergic reactions. On the basis of previous research (Noort et al., 1999), it is likely that the cysteine-34 residue will react readily with the activated glucuronides, but whether the resulting thioester adduct is stable enough to accumulate in the organism, is not very plausible. However, Grillo et al (2003; see also Grillo and Hua, 2003 for corresponding adducts of NSAID zomepirac) found that the S-glutathione adduct of the NSAID diclofenac was excreted from the bile of rats after administration of diclofenac glucuronide (see also Li et al, 2002).

Protein binding of glucuronides of benzoic acids that are structurally related to 3-PBA has been reported (see, *e.g.*, Akira et al., 2002). In more general terms, it appears that the degree of covalent binding to proteins of acidic drugs in man correlates well with the chemical reactivity of the glucuronides of these drugs (Benet et al, 1993). We envisaged that adduct formation with proteins of glucuronide derivatives of carboxylic acid metabolites of permethrin is probable and will provide a useful biomarker to assess cumulative exposure to this pyrethroid. We first selected some model compounds to explore whether the obtained acyl glucuronide are indeed electrophilic compounds.

Adducts of 3-PBA glucuronide

Several adducts resulted upon incubation of 3-PBA-glucuronide with glutathione, Z-Lys and the model peptides ASSAKQR and LKZASLQK, with Z = S-carboxymethylcysteine. The peptides have been derived from human serum albumin; the lysine (K) residues have been reported to be reactive towards acylating reagents (*e.g.*, Noort et al., 2000) and also towards glucuronides (*e.g.*, Ding et al, 1995). Characteristic for all incubations with 3-PBA-glucuronide is that the resulting adducts either show addition of 196 amu ($-H + 3$ -phenoxybenzoic acid $-OH$) or of 372 amu ($-H + 3$ -PBA glucuronide $-H_2O$), resulting from direct acylation or by reaction through the glycation mechanism.

In case of glutathione the binding site is the SH group, as could be assessed by the MS-MS spectrum of the adduct; adduct formation had occurred through the transacylation mechanism. See Figure 10 for the chemical structure of the adduct and Figure 11 for the MS-MS spectrum. There was also some evidence that an adduct had been formed through the glycation mechanism. Although the expected mass of such an adduct was detected, we could not entirely solve the obtained tandem MS spectrum. This will be studied in more detail in the next year of the grant period.

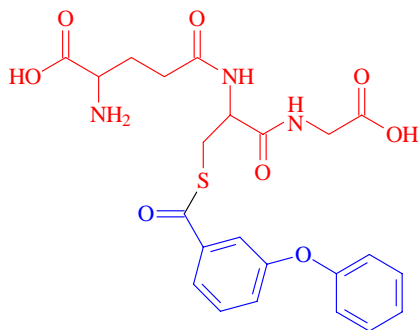


Figure 10. Chemical structure of glutathione 3-PBA glucuronide adduct

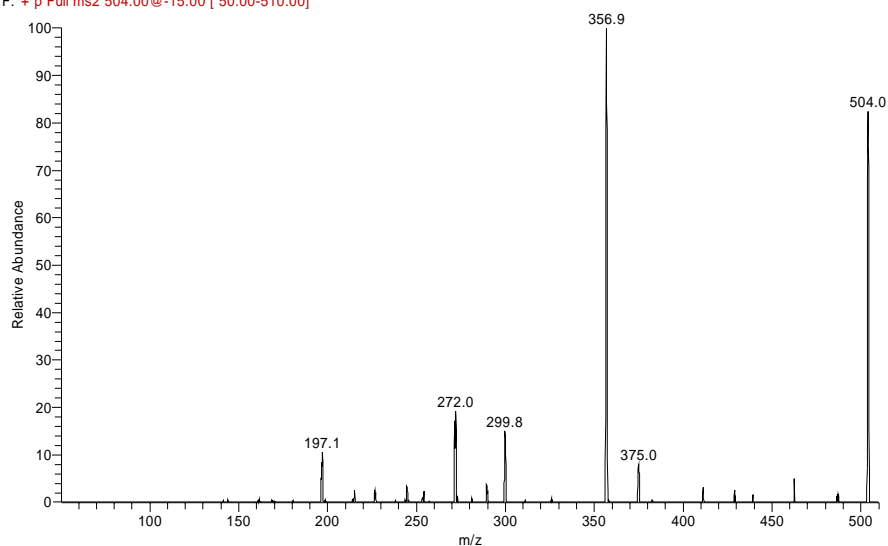


Figure 11. Tandem ES(+) MS spectrum of 3-PBA glucuronide adduct to glutathione. Typical fragments for 504.0 $[M + H]^+$: 375.0 $[y_2'']$, 356.9 $[y_2'' - H_2O]$, 299.8 $[C(O)-NH-C=C-S-C(O)-3\text{-phenoxyphenyl}]^+$, 272.0 $[H_3N-C=C-S-C(O)-3\text{-phenoxyphenyl}]^+$, 197.1 $[C(O)-3\text{-phenoxyphenyl}]^+$

In case of Z-Lys-OH the binding site is the NH_2 group of lysine; the only observed adduct with m/z 477 $[M+H]^+$ was the adduct that had resulted from transacylation.

In case of incubation of 3-PBA glucuronide with ASSAKQR, transacylation adducts could be detected that were either derived from modification of the free amino group at the N-terminus, or from modification from the ϵ -amino group in lysine. We focused on the latter adduct; see Figure 12 for mass spectra.

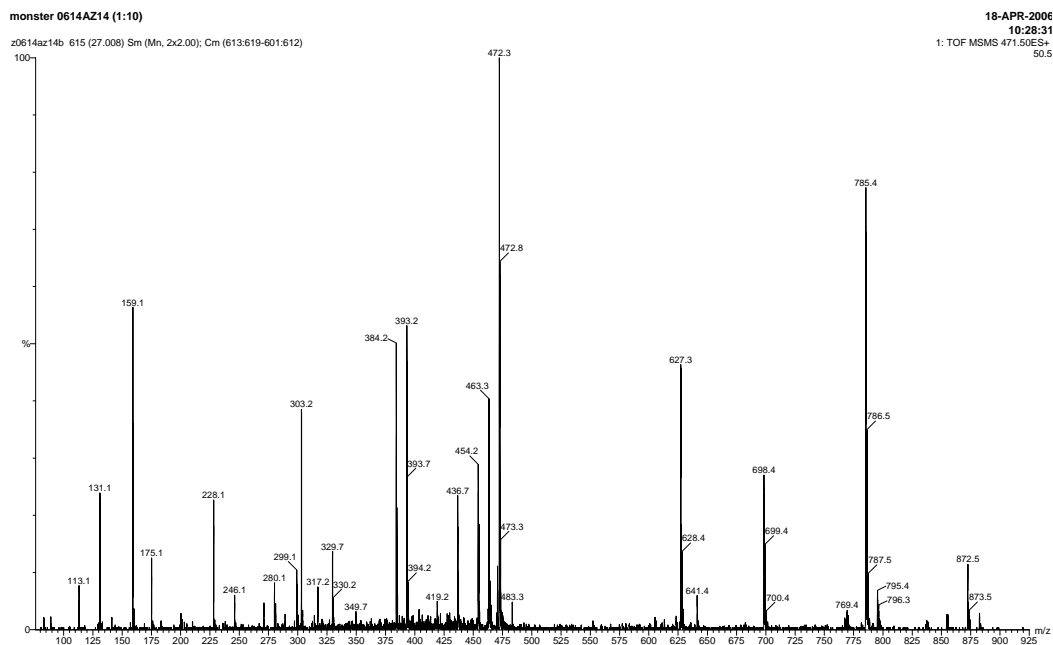


Figure 12. Tandem MS spectra of adduct of 3-PBA glucuronide with ASSAKQR, formed by the transacylation mechanism. Characteristic fragments: 872.5 [$y_6'' + 196$], 785.4 [$y_5'' + 196$], 698.4 [$y_4'' + 196$], 627.3 [$y_3'' + 196$], 472.3 [MH_2^{2+}].

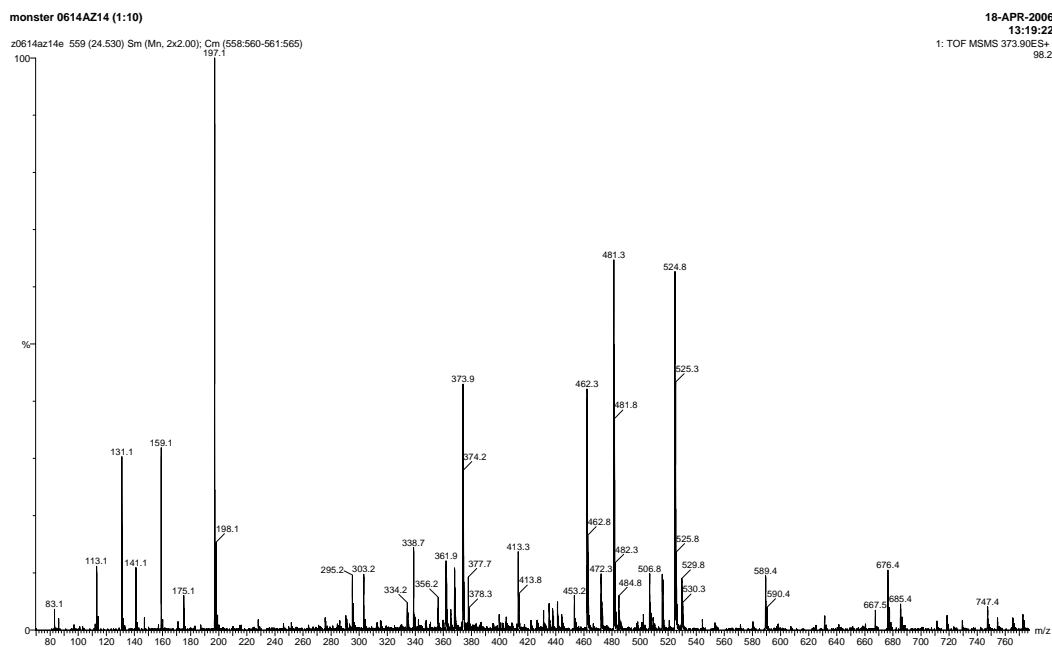


Figure 13. Tandem MS spectra of adduct of 3-PBA glucuronide with ASSAKQR, formed by the glycation mechanism. Characteristic fragments: 524.8 [$y_6'' + 372.1$], 481.3 [$y_5'' + 372.1$], 373.9 [MH_3^{3+}], 197.1 [$C(O)$ -3-phenoxyphenyl] $^+$.

In case of the model peptide LKZASLQK similar adducts were observed, formed by either the transacylation mechanism, or by the glycation mechanism.

In case of incubation of 3-PBA glucuronide with human plasma followed by pronase digestion of isolated albumin, the adduct derived from the tripeptide Cys-Pro-Phe containing the reactive Cys-34 residue, could not be observed. Probably this adduct is too unstable to survive the incubation with pronase; evidence for the lability of an S-acylated adduct was already obtained in our experiments with glutathione. After trypsin digestion of the albumin from the exposed plasma, no ASSAKQR adducts could be detected. However strong evidence was obtained for the formation of adducts, through either the transacylation or glycation mechanism, with LKZASLQK. In both cases, strong ion fragments were observed with m/z 197. Unfortunately, we could not yet identify unambiguously the site of modification; this will be explored in more detail in the second annual period.

Adducts of Cl_2CA -glucuronide

Incubation of glutathione with Cl_2CA -glucuronide resulted in the formation of a similar adduct as was observed for 3-PBA glucuronide, i.e., by modification at the thiol function; see Figure 13 for the ES+ tandem MS spectrum.

Adducts of Cl_2CA -glucuronide with Z-Lys-OH could not be detected; this might indicate that this particular type of acyl glucuronide is less reactive.

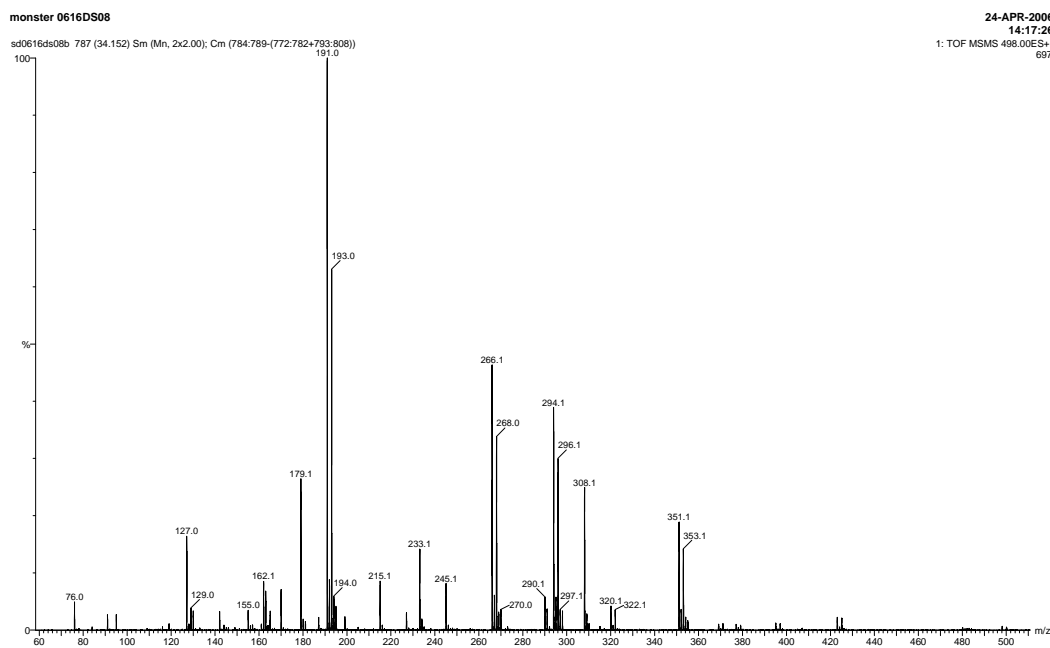


Figure 14. Tandem ES(+) MS spectrum of Cl_2CA -glucuronide to glutathione. Typical fragments for 498.0 $[M + H]^+$: 351.1/353.1 $[y_2'' - H_2O]$; 294.1/296.1 $[C(O)-NH-C=C-S-C(O)-dichlorovinyl-dimethylcyclopropyl]^+$; 266.1/268.1 $[H_3N-C=C-S-C(O)-dichlorovinyl-dimethylcyclopropyl]^+$; 191.0/193.0, Cl_2CA fragment.

V APPENDIX

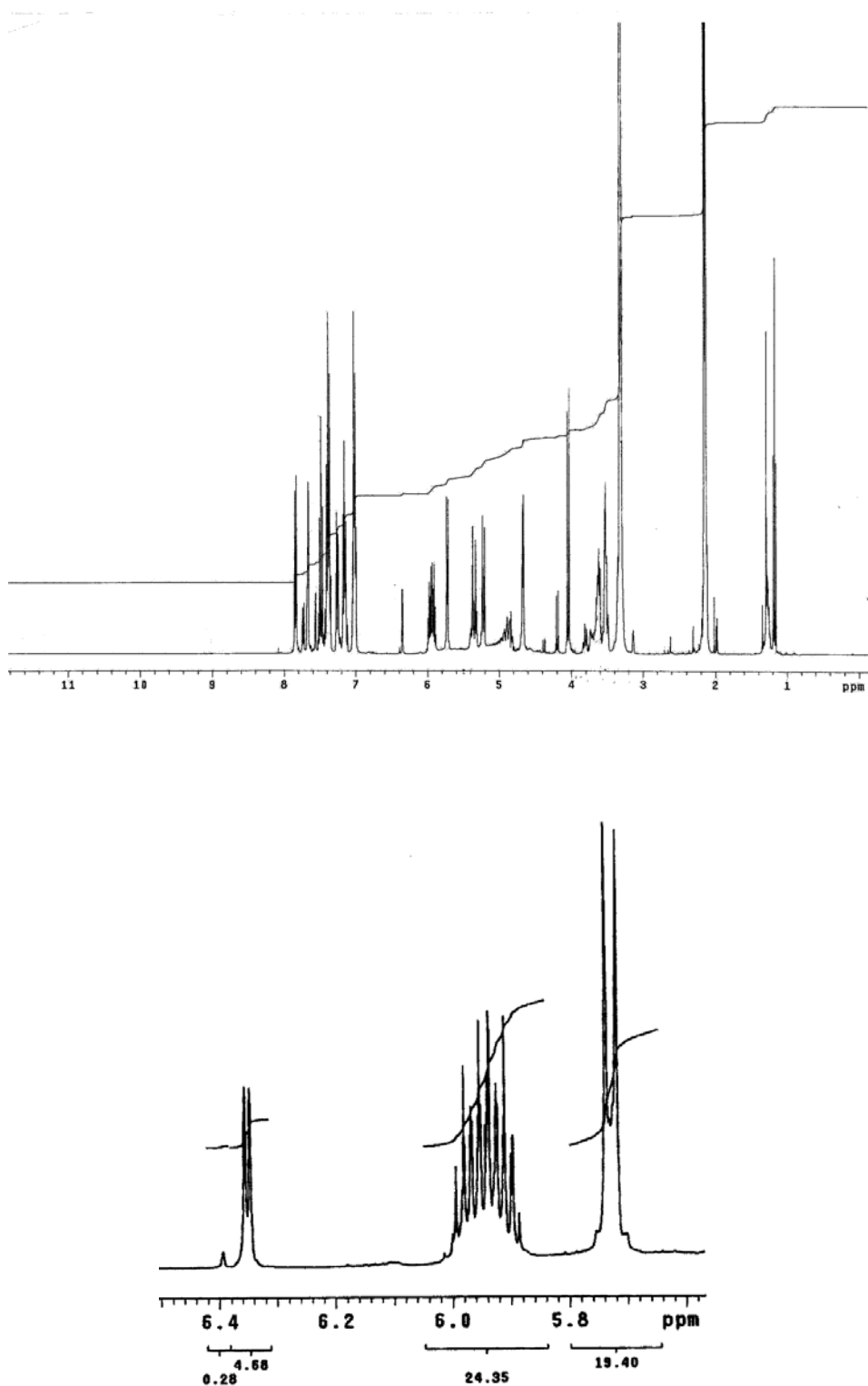


Figure 1A. ¹H-NMR spectrum of anomeric mixture of allyl 1-O-(3-phenoxybenzoyl)-D-glucopyranuronate (2). Upper panel: entire 400 MHz ¹H-NMR spectrum. Lower panel: part of the spectrum, showing the H1-α around 6.4 and H1-β around 5.7. The multiplet around 5.9 – 6.0 is part of the allyl group.

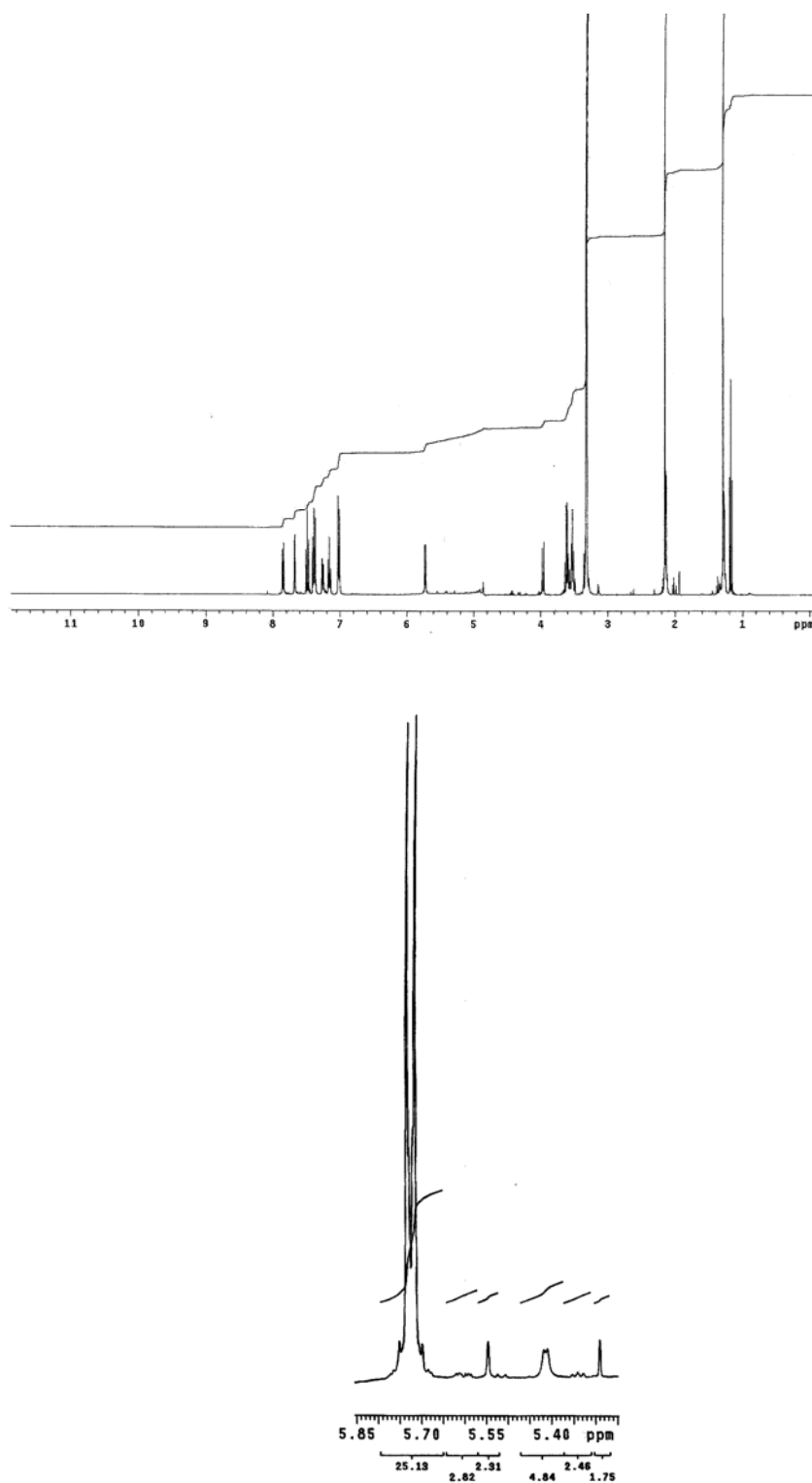


Figure 2A. ^1H -NMR spectrum of anomeric mixture of 1-O-(3 phenoxy-benzoyl)-D-glucopyranuronate (3; 3-PBA-glucuronide). Upper panel: entire ^1H -NMR spectrum. Lower panel: part of the ^1H -NMR spectrum, showing the characteristic doublet of H1- β around 5.7.

VI KEY RESEARCH ACCOMPLISHMENTS OBTAINED IN THIS GRANT PERIOD

1. The synthesis of the β -acyl glucuronide of the permethrin-derived metabolite 3-PBA has been successfully accomplished, after exploring two different synthetic routes.
2. The synthesis of the acyl glucuronide of the permethrin-derived metabolite Cl_2CA has been accomplished, although as yet the α - and β -anomers cannot be separated.
3. The reaction of 3-PBA glucuronide with glutathione has been shown to result in an (unstable) S-acylated adduct.
4. The reaction of 3-PBA glucuronide with model compounds has been shown to result in the formation of both adducts formed by transacylation, as well as adducts formed by the glycation mechanism.
5. For Cl_2CA glucuronide, only adducts with glutathione could be detected, derived from transacylation. No adducts could be observed after incubation of Cl_2CA glucuronide with amino-containing model compounds
6. Preliminary experiments with human plasma have been carried out. After incubation of 3-PBA glucuronide with human plasma, followed by sample work-up, two peptide adducts could be detected that were derived from albumin. In the next annual period, this will be studied in more detail.

VII REPORTED OUTCOMES

Publications

none

Presentations

Generic assays for exposure biomonitoring of alkylating agents

NATO TG-009

Natick, MA, USA, October 2005

Presented by D. Noort

Persistent biomarkers of exposure

2nd International Workshop on Environmental and Health Hazards,
The Hague, The Netherlands, April 5&6, 2006

Presented by D. Noort

Persistent biomarkers of exposure of potentially neurotoxic compounds

23rd International Neurotoxicology Conference

Little Rock, USA, September 17-21, 2006

Presented by D. Noort

VIII CONCLUSIONS

1. Synthesis of β -acyl glucuronide is not a straightforward task.
2. Adduct formation of the intrinsically reactive acyl glucuronide of the permethrin metabolite 3-PBA with various model amino acid and peptide model compounds has been assessed.
3. Thus far, adduct formation of the similar acyl glucuronide of the permethrin metabolite Cl₂CA could only be seen with glutathione.
4. Upon incubation of 3-PBA glucuronide with plasma, strong evidence was obtained for adduct formation with human serum albumin.
5. From the reported in vitro results it can be concluded that adduct formation of permethrin metabolites is highly likely to occur in vivo as well. Since in general albumin adducts have a rather long half life time (weeks), the identified adducts might form the basis for a novel biomonitoring methodology for exposure to permethrin.

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None

Meeting Abstracts

Persistent biomarkers of exposure

D. Noort

Abstract book 2nd International Workshop on Environmental and Health Hazards,
The Hague, The Netherlands, April 5&6, 2006

Persistent biomarkers of exposure of potentially neurotoxic compounds

D. Noort

Abstract book 23rd International Neurotoxicology Conference
Little Rock, USA, September 17-21, 2006

XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE
AGREEMENT

Dr. D. Noort

Dr. B.L.M. van Baar

Mr. A. Fidder

Mr. A.G. Hulst

Mr. S.H. van Krimpen

Mrs. A. Van Zuylen